A modern workflow for non-destructive DNA extraction and slide preparation of thrips (Insecta, Thysanoptera) for taxonomic studies and collection deposition

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Recent studies of taxonomy, systematics and ecology often depend on molecular data, and non-destructive DNA extraction protocols have gained popularity as a method of saving a physical voucher specimen. However, the quality of the permanently mounted specimens is seldom discussed, and detailed protocols often left out. Here a modified and optimized protocol for Thysanoptera is presented and outlined in detail.

Key words: Preparation, Thysanoptera, DNA extraction, morphology, non-destructive.

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Introduction

The order Thysanoptera Haliday, 1836 are more commonly known as thrips, and are minute insects often not larger than 3 mm in length. The larger species may reach a length of up to 5 mm. They are found within flowers, seed pods, on bark or in soil and deadwood, feeding on pollen, spores, fungi, algae, or prey (Kirk 1996). The small size and secluded lifestyle might be reasons for the relatively few taxonomic studies of the order. Some has caught the attention not only from researchers but also from the commercial and private sector, due to some species that are considered as pests in agriculture (Paine 1992) and even as invasive (Held *et al.* 2003, Boyd & Held 2006).

Certain species determination is dependent on morphological studies of microscopic characters, such as bristle shape and length, mouth parts and other both external and internal structures. The preparation of specimens on microscope slides generally follows an established principle and workflow. As outlined by Kirk (1996) this involves first storing the freshly collected specimen in an 60% AGA solution (ethanol, glycerol, and acetic acid in 10:1:1 proportions), followed by soaking in 60% ethanol. The ethanol is replaced by a 5% solution of a base, often sodium hydroxide or potassium hydroxide, the specimen is punctured and massaged and left in the solution until macerated and clear. The base is replaced with distilled water and thereafter the specimen is treated through an ethanol ladder up to 99.5% ethanol. Finally, the specimen is further cleared in essential oil, such as clove or cedarwood oil, before mounting in suitable medium on a microscopic slide. This process has been modified several times, e.g., by skipping some ethanol washing steps or replacing the bases and acids with more commonly available components. A few of these are exemplified in Bisevac (1997) and Kobro (2013).

The process of maceration and clearing using acids and bases has the negative effect that it

also breaks down DNA and makes it impossible to extract it for sequencing. Sequencing of DNA has become standard procedure in many cases, with reference libraries for molecular barcoding as well as species identification methods using DNA being important steps in both taxonomical and ecological work. The sequences may not only be used in species delimitation contexts, but also in e.g. population and evolutionary studies, hence even already sequenced species can provide additional invaluable molecular data if collected from various geographical areas as well as throughout time.

As morphological species determinations often require both preparation and skilled personnel, DNA extraction and barcode sequencing has become a more prioritized step in specimen treatment after collection. To verify species determinations non-destructive DNA extraction protocols have been developed to meet the requirements of both speedy molecular data processing and high-quality morphological identification. In non-destructive DNA extraction the whole specimen is submerged in lysis buffer with proteinase K, the soft tissue is dissolved, and the sclerotized parts retained and transferred to e.g. 70-80% ethanol or directly prepared for microscopic studies (see e.g. Porco *et al.* 2010, Miura *et al.* 2017, Marquina *et al.* 2022). The specimen can thereafter be used for photography and taxonomic and systematic studies. A review of and analysis of different non-destructive protocols are given in Marquina *et al.* (2022). For Thysanoptera specifically non-destructive protocols have been used by e.g. Buckman *et al.* (2013) and Kumar *et al.* (2014).

During the work on the project of describing and identifying the Swedish thrips fauna, starting with the family Phlaeothripidae (Wahlberg & Gertsson 2022), non-destructive DNA extraction has been an essential tool to gathering data and at the same time preparing many specimens for preparation and mounting. We noticed some issues following extraction and mounting that are not thoroughly documented and discussed in previous studies and protocols. The main issues were: 1) insufficient clearing and bleaching of cuticle and 2) residual salts leaking from mounted specimen. The insufficient bleaching of the cuticle makes it difficult to study internal and ventral structures, as well as cuticular patterns and setation. All characters important in species determination. The presence of residual salts causes leakage into the surrounding medium of the mounted specimens, rendering the specimen a "dirty" appearance and making it unsuitable for photographic applications. Herein we present a workflow for non-destructive DNA extraction and mounting of lysed specimens modified and optimized for Thysanoptera.

Material and methods

To demonstrate the results of different approaches, 2 specimens of 2 species were treated with a standard non-destructive DNA extraction protocol and 2 other specimens of the same species were treated with the modified protocol for Thysanoptera. The first species was *Chirothrips manicatus* Haliday, 1836. The second species for each protocol was especially dark and sclerotized, *Cephalothrips monilicornis* (Reuter, 1880), making it suitable to demonstrate the bleaching of the cuticle for visualizing internal structures. All specimens were collected and stored in 80% ethanol before extraction. Lysis buffer and proteinase K were from the KingFisher Cell and Tissue DNA Kit (Thermo Scientific). The standard protocol is adapted from Buckman *et al*. (2013).

Standard non-destructive DNA extraction and mounting protocol

1. Whole specimen was placed in 200 µl of lysis buffer and 25 µl of proteinase K. Lysis was performed overnight, with gentle shaking in 56°C.
2. The lysed specimen was transferred to 99.5% ethanol.
3. The specimen was placed in a drop of Euparal on coverslip, which was transferred to a microscope slide by lowering the slide onto the coverslip and turning it over as soon as it touches the medium. The slide was left to dry.

Modified DNA extraction and mounting protocol for Thysanoptera

1. Whole specimen was placed in 200 µl of lysis buffer and 25 µl of proteinase K. Lysis was performed overnight, with gentle shaking in 56°C.
2. The lysed specimen was transferred back to 80% ethanol and left for 72 hours.
3. The ethanol was removed, together with any residual salts, and replaced with new 80% ethanol and left for 24 hours.
4. 80% ethanol was replaced with 95% ethanol for 15 minutes.
5. 95% ethanol was replaced with 99.5% ethanol for 5 minutes.
6. The clarity of the cuticle was inspected, in the case of too dark and opaque appearance the specimen was placed in clove oil for up to 45 minutes (until sufficiently clear).
7. The specimen was placed in a drop of Euparal on coverslip, which was transferred to a microscope slide by lowering the slide onto the coverslip and turning it over as soon as it touches the medium. The slide was left to dry.

Results

The specimens that had been treated with the standard non-destructive DNA extraction and mounting protocol showed residual salt particles, increasing in visibility over the following days after mounting (Figure 1A). The more sclerotized *C. monilicornis* had extensive dark pigmentation left as well as being too dark for any internal structures as well as surface patterns to be visible in light microscope (Figure 2A).

The specimens treated with the modified protocol kept their clear and clean appearance over the following days, without any residual salts visible (Figure 1B). There was salt present in the 80% ethanol before transfer to clean ethanol. The dark and sclerotized specimen that was treated with an extra step in clove oil showed a brighter and clearer results, making the visualization of both internal structures and surface patterns possible (Figure 2B).

As the protocol does not include alterations of the lysis and DNA extraction process, the DNA yield was unaffected.

Discussion

With novel molecular methods such as DNA barcoding, metabarcoding and whole genome sequencing being standard in many systematic and ecological studies today there is a very high urgency in traditional morphology-based taxonomy to keep the pace in methodology

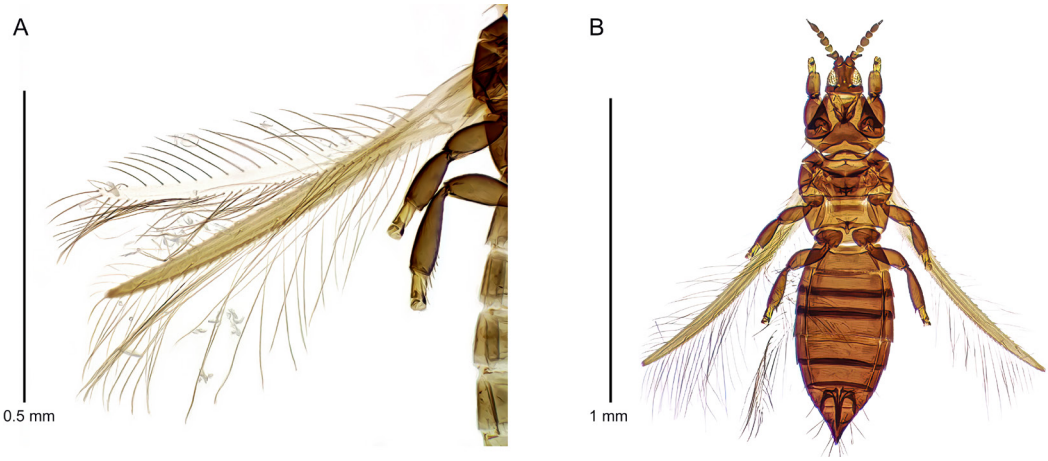

FIGURE 1. *Chirothrips manicatus*. **A**. Residual salts caught in fringes of wing. **B**. Dorsal view of habitus, specimen treated with modified protocol to remove residual buffer salts.

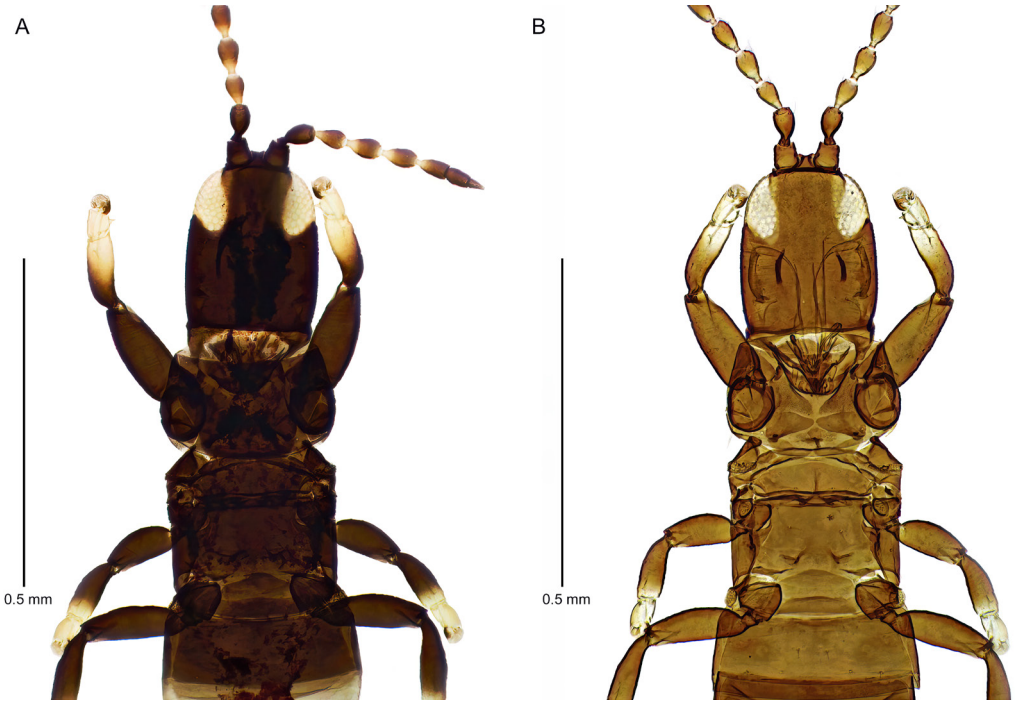

FIGURE 2. Dorsal view of part of *Cephalothrips monilicornis*. **A**. Specimen without additional treatment with clove oil, prepared directly from extraction and 99.5% ethanol. **B**. Specimen treated with modified protocol and additional step with clove oil.

and protocol development. Voucher specimens from DNA extraction can with appropriate methods be stored in the same high quality and permanency as specimens treated using traditional methods, facilitating expert determinations and morphological data to be stored alongside genetic data.

However, there are problems with insufficient references to protocols used in many studies, only short mentions of the methods used without a detailed description. This makes reproducibility impossible. Furthermore, improper preparation, mounting and storage of vouchers leads to the loss of the physical specimen. Reference DNA databases without connection to physical collections is a problem in making sure of correct species identification. Vouchers and types should be clear and perfectly mounted and keep their appearances over time.

The method herein describes a protocol for properly clearing and preparing thrips specimens for mounting, with the aim of morphological studies and long-term storage and collection deposition. Even though this method significantly lengthens the process from extraction to finished slide, it is important that vouchers are permanently fixed in a as pristine and usable state as possible. The lysis process can still not fully replace additional lightning of the cuticle in some cases. This protocol is an important part in bringing modern molecular methods and traditional morphology-based taxonomy together.

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